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CALCIUM AND POTASSIUM CURRENTS OF THE MEMBRANE OF A BARNACLE MUSCLE FIBRE IN RELATION TO THE CALCIUM SPIKE

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SUMMARY

1. The behaviour of membrane currents in a giant muscle fibre of a certain barnacle *Balanus nubilus*, Darwin, was studied by using voltage clamp technique after treating fibres with a Ca-chelating internal solution.

2. Membrane currents can be classified into an early transient current and a late outward current.

3. The early transient current can be considered a sum of an inward and an outward component of different properties.

4. A conditioning depolarization suppresses the inward component but not the outward component.

5. Procaine suppresses the outward component and Co ions suppress the inward component.

6. Changes in the external Ca ion concentration alter the inward component but not the outward component.

7. The inward component is considered to be carried by Ca ions and the outward component mainly by K ions.

8. The outward component of the early current is similar to the current expected from the resting membrane conductance.

9. It is concluded that the early conductance increase of the barnacle muscle fibre membrane occurs only to Ca ions but not to K ions, although the membrane potential at the peak of spike is determined by both ions.

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INTRODUCTION

It has been shown in a number of excitable tissues that the action potential is produced by an increase in the conductance of the membrane to Na ions followed by an increase to K ions (Hodgkin & Huxley, 1952*a*). However, in certain other tissues such as crustacean muscle fibres, the active inward current is carried by Ca ions instead of Na ions (Fatt & Ginsborg, 1958; Hagiwara & Naka, 1964). Voltage clamp type analyses show that the electrical behaviour of the first type membrane can successfully be described with a model of two independent membrane conductances, the early or Na-conductance and the late or K-conductance (Hodgkin & Huxley, 1952*c*). The present work was undertaken to analyse the behaviour of the second type membrane in a giant muscle fibre of a barnacle, *Balanus nubilus*, Darwin, using voltage clamp techniques. The object of the work was first to prove the existence of a dual conductance system, and secondly to examine the details of the K-conductance mechanism during the early stages when the Ca channel is open.

METHODS

Materials and preparations. Giant muscle fibres of a barnacle, *Balanus nubilus*, Darwin, were used. Specimens were obtained from the Pacific Coast of California. Muscle fibres had a diameter of 0·5–2·0 mm. The method of isolation of single muscle fibres was similar to that described previously (Hagiwara & Naka, 1964).

Recording potentials and voltage clamp. An isolated fibre was placed on a lucite platform and an internal solution was injected through a longitudinally inserted glass pipette as described by Hagiwara & Naka (1964). After the injection, the pipette was withdrawn and a longitudinal double wire (silver) electrode was introduced. One wire, used for current injection, had a diameter of 350 μ and was insulated except for a final stretch of 1·7 cm, which was platinized. The other wire, used for potential recording, was 60 μ in diameter and insulated except for a stretch of 2 mm centred in the platinized region of the first wire. The bare region of the second wire was chlorided. The lucite platform was separated by partitions into three compartments of 0·5, 0·7 and 1·0 cm length (see the diagram of Fig. 1). The fibre extended from compartment to compartment through notches made in these partitions and the space between the fibre and the wall of the partition at the notch was sealed with Vaseline. The longitudinal wire electrode was so inserted that the central 7 mm of the platinized portion of the current wire was in the central compartment (*b*). Pairs of electrodes made of a large number of fine chlorided silver wires (maximizing surface area) were placed in each compartment. All three compartments were filled with saline and the membrane current from the portion of the fibre in the central compartment was recorded as an IR drop across a 10 Ω resistor inserted between the electrodes in the saline and ground. The electrodes in the other two compartments were directly grounded. In the central compartment a fine silver wire (100 μ), insulated except for a 2 mm chlorided portion at the tip, was placed just outside the fibre opposite the uninsulated portion of the internal recording wire. Potential changes across the membrane were recorded as a potential difference between these electrodes. The absolute value of the resting membrane potential was occasionally

observed by introducing 3 M-KCl filled glass micropipettes. The feed-back system for the voltage clamp is shown diagrammatically in Fig. 1 and is similar to that employed by Hagiwara, Takahashi & Junge (1967).

Records *B* in Fig. 1 shows the membrane currents (upper trace in each record) associated with a rectangular membrane potential change (hyperpolarization, lower trace) obtained with voltage clamp technique. These records show that the capacitative current has a long tail which may be due to delay in the equivalent resistance-capacity circuit of the internal tubular system. This tubular system has been described by Hoyle & Smyth (1963), and it may be similar in its electrical behaviour to that of the frog (cf. Falk & Fatt, 1964). The tail current often overlapped the early phase of the membrane current associated with excitation. In order to record membrane current without being seriously disturbed by the tail capacity current, the

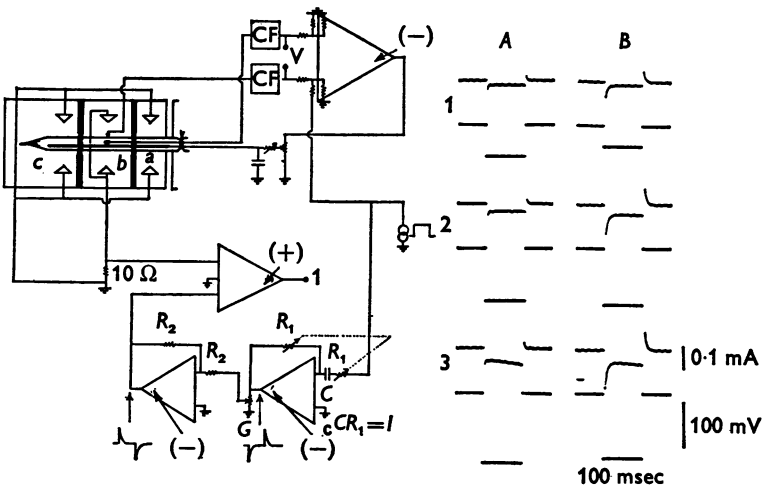

Fig. 1. Diagram of experimental arrangement. See text. *A* and *B*, membrane currents (shown by the upper trace in each pair) associating with negative shifts of the membrane potential (the lower trace) from the resting potential level obtained by using the voltage-clamp technique with (*A*) and without (*B*) the compensation circuit for the tail capacity current.

following circuit (Fig. 1) was introduced. A voltage pulse identical to the commanding pulse for the voltage clamp was fed to a circuit of variable time constant ($I = CR_1$) and attenuation. This output was inverted and fed to the second input of a differential amplifier, while its first input was connected to the output of the current recording circuit. The time constant and the attenuator were adjusted for a given commanding voltage pulse so that the long-lasting tail capacity current was reduced to a minimum. Since the tail capacity current was linear with the amplitude of the commanding voltage pulse this gave an approximate balance for any commanding pulse. Records *A* of Fig. 1 were obtained from the same fibre as that used to obtain records *B* but with the tail capacity current compensated in this way.

Solutions. Compositions of external solutions used are listed in Table 1. Variable Ca concentration in high Mg (100 mM) was obtained by mixing Ca-Mg rich saline and Ca-free Mg-rich saline in an appropriate proportion. Procaine hydrochloride and $CoCl_2$ were dissolved in the saline (to make the saline slightly hypertonic). The composition of the internal solution was: KOH, 400 mM: EGTA (ethyleneglycol

bis(aminoethylether)-N,N′-tetra-acetic acid), 100 mM; Tris-maleate, 20 mM; sucrose, 340 mM and methane-sulfonic acid 184 mM (pH = 7·0). All the experiments were performed at low temperature (8–10° C) by circulating ice water beneath the lucite platform on which the muscle fibres were kept.

RESULTS

Membrane currents associating with rectangular potential changes

Figure 2*B* shows an action potential of a barnacle muscle fibre obtained after intracellular injection with the EGTA containing solution. The rest of the records in the Figure (Fig. 2*A*) show membrane currents of the same fibre obtained by voltage clamp technique. The membrane current is characterized by an early transient inward current followed by a late outward current which developed to a steady state after 30–50 msec (9–10° C).

TABLE 1. Compositions of external solutions (mM)

	NaCl	KCl	$CaCl_2$	$MgCl_2$	TM*	TOH*	HCl
Normal Na-saline	461·5	8	20	12	10	—	—
Normal Tris-saline	—	8	20	12	—	505	371
Ca-Mg rich saline	—	8	200	100	—	82·5	56·5
Ca-free Mg-rich saline	—	8	—	100	—	435	305

* TM = Tris-maleate and TOH = Trizma base. pH of solutions, 7·7.

The early inward current appeared at −40 to −35 mV and rapidly increased its amplitude to a maximum with positive shift of membrane potential up to about −10 mV. With further positive shift the amplitude decreased and the current reversed its sign from inward to outward at a certain membrane potential. This reversal potential was close to the potential at the peak of the action potential obtained without voltage clamp. The result in Fig. 2 was obtained in normal Tris-saline. Essentially the same result was obtained in normal Na-saline. These membrane currents are similar in behaviour to those already observed in other excitable tissues, such as squid giant axons where the early transient and the steady-state currents are identified as being the Na and K currents under normal conditions (Hodgkin & Huxley, 1952*a*).

Effects of conditioning voltage pulse

Figure 3*A* shows the current–voltage relations of the membrane at the peak of the early transient current (filled circles, continuous line) and at the steady state (interrupted line) when the holding membrane potential was

-70 mV and the fibre was in the normal Tris-saline. The other curves were obtained with conditioning voltage pulses. In these experiments the membrane potential was displaced from the holding level to a conditioning potential level for 780 msec, and then given a test pulse (Fig. 3*B*). The current–voltage relations at the peak of the early transient current was unaltered by the conditioning pulse when the membrane potential during the conditioning pulse was more negative than about -45 mV (see rela-

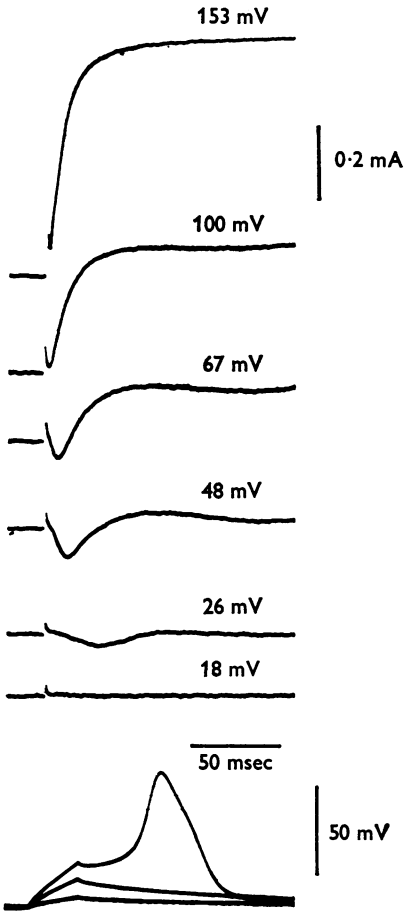

Fig. 2. Membrane currents associated with positive shifts of the membrane potential from the resting potential (-50 mV). A figure listed to each trace indicates the amplitude of potential shift measured from the resting potential. The lowermost record shows an action potential (upper trace) produced by a short constant current pulse in the absence of voltage clamp. External saline, normal Tris-saline. Treated with the Ca-chelating internal solution. Temperature 9° C.

tions obtained at −97 mV and illustrated with open circles in Fig. 3*A*). The amplitude of the early inward current for a given test pulse became smaller when the conditioning membrane potential was more positive than about −45 mV. Relations illustrated with filled triangle and open squares were obtained at conditioning membrane potentials of −30 and −16·5 mV

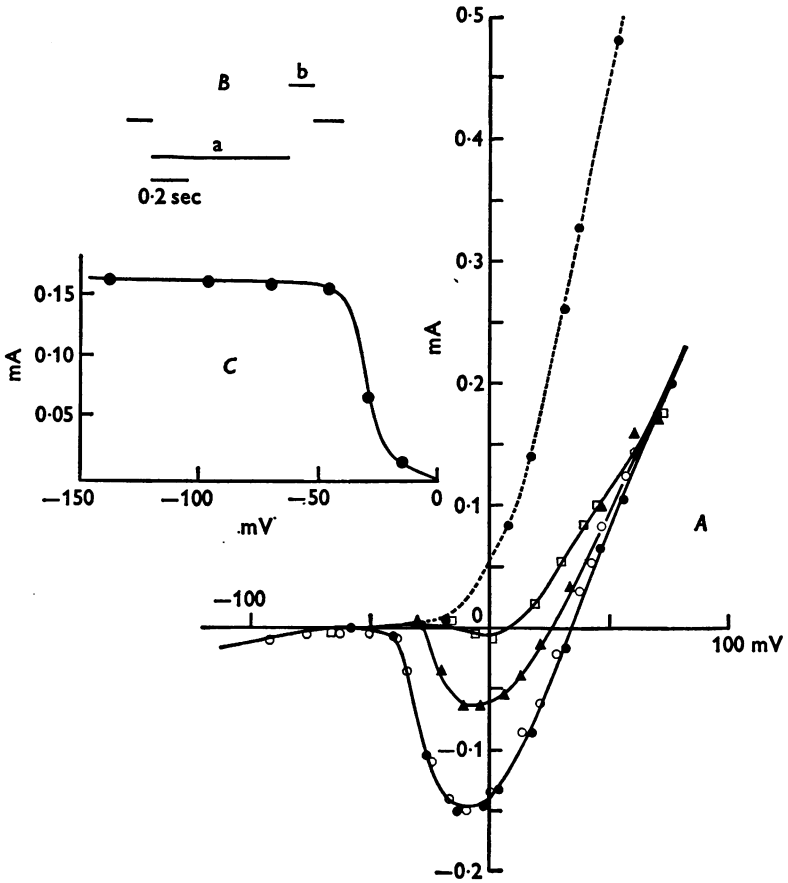

Fig. 3. *A*. Current–voltage relations at the peak of the early transient current (continuous lines) and at the steady state (interrupted lines, obtained at 125 msec after the onset of the test voltage pulse). Relations illustrated with filled circles are the control obtained at the holding membrane potential of −70 mV. Those illustrated with other symbols (open circle, −97·5 mV; filled triangle, −30 mV; open square, −16·5 mV) were obtained from the same fibre when the test pulse (*b* in the inset *B*) was preceded by conditioning voltage pulse of 780 msec (indicated by *a* in the inset *B*). Steady-state relations obtained with conditioning were identical to the control. *C*, relationship between the maximum amplitude of the early inward current (ordinate) and the membrane potential at the conditioning voltage pulse (abscissa). The external solution, normal Tris-saline. Treated with the Ca-chelating internal solution. Temperature 7° C.

respectively. The inward current was much reduced in the former and became barely observable in the latter. In order to illustrate the relation between the early inward current and the conditioning membrane potential, the maximum value of the peak inward current found in each current–voltage relation was plotted against the conditioning membrane potential (Fig. 3*C*).

This behaviour of the early inward current is very similar to that found for the inactivation of the Na current in the squid giant-axon produced by conditioning voltage pulses (Hodgkin & Huxley, 1952*b*). In the squid giant axon, however, not only inward but also the outward Na currents were suppressed by the conditioning depolarization whereas no suppression was seen for the early transient outward current in a barnacle muscle fibre. The amplitude of an early outward current with a conditioning depolarization did not become smaller than the control value even at relatively large positive membrane potentials. In a few experiments the current–voltage relation was observed for the range of membrane potentials up to +200 mV with and without conditioning (Fig. 6). Even over this range the two relations did not cross each other. These results indicate that early outward currents observed at large positive membrane potentials are insensitive to conditioning depolarizations even when the same conditioning suppresses the early inward current significantly.

The foregoing results suggest that the early transient current represents the sum of two component currents, one being sensitive and the other insensitive to conditioning depolarization. Experiments reported previously (Hagiwara & Naka, 1964; Hagiwara, Chichibu & Naka, 1964) have shown that the membrane potential at the peak of the spike in a barnacle muscle fibre is determined by the external Ca^{2+} and the internal K^+ concentrations. This suggests that the early transient membrane current is carried by Ca and K ions. The internal Ca ion concentration is very small (less than 10^{-8} M, Hagiwara & Nakajima, 1966*a*) compared with that of the external solution because of the internal injection of EGTA. In contrast to this the K ion concentration is low outside (8 mM) and high inside, since the internal injecting solution contained 450 mM-K. Therefore, in the range of membrane potentials and in the present study, Ca ions move almost exclusively inward through the membrane, K ions mainly outward. Under these conditions the early transient membrane current should represent the sum of the inward Ca current and the outward K current. They should be equal in amplitude but have opposite signs at the reversal potential. An increase in the amplitude of the early inward current should be due to a decrease in the outward K current and/or an increase in the inward Ca current. The experimental results described above, then, suggest that the phenomenon similar to the inactivation observed for the Na current in a

squid giant axon is found only for the early Ca current but not for the early K current in the barnacle muscle fibre.

Effects of procaine and cobalt ions on the membrane current

A small amount of procaine in the external solution increases the overshoot as well as the duration of the spike potentials of fibres injected with a Ca-chelating solution (Hagiwara & Nakajima, 1966*b*). This effect was more marked when applied to fibres untreated with Ca-chelating agent. Those

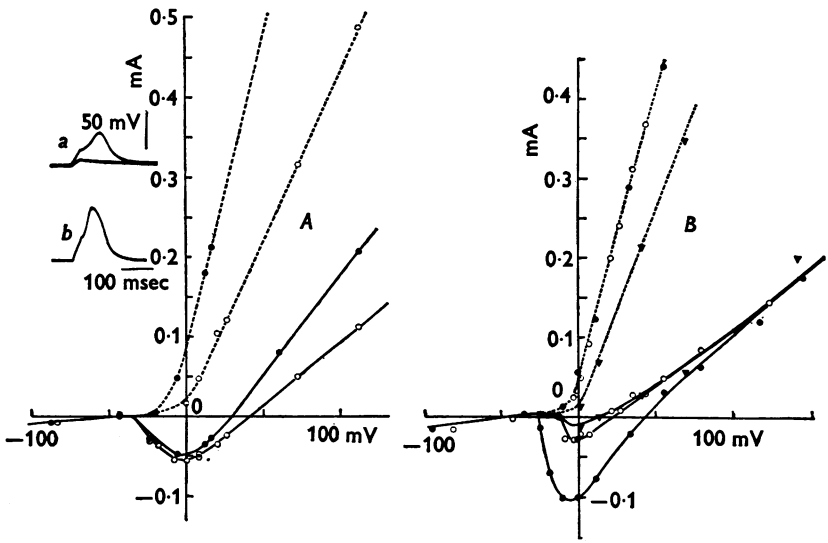

Fig. 4. Current–voltage relations at the peak of the early transient current (continuous lines) and at the steady state (interrupted lines, obtained at 300 msec from the onset of the test pulse). *A*, effect of procaine. Filled and open circles show results obtained before and after the application of 0·05 % (w/v) procaine in normal Tris-saline. Inserts *a* and *b* show spike potentials obtained before and after procaine. No Ca-chelating solution was injected. Temperature 7° C. *B*, effect of Co ions. Filled circles, open circles and filled triangles are the data obtained in the same muscle fibre at the resting potential (−50 mV, control, filled circles), with conditioning depolarization (136 msec at −13 mV, open circles) and with 30 mM-$CoCl_2$ (filled triangles) respectively. External solution, normal Na-saline. Treated with the Ca-chelating internal solution. Temperature 8° C.

fibres usually do not produce all-or-none spike potentials at room temperature. Procaine usually makes those fibres capable of producing all-or-none spike potentials (see Fatt & Katz, 1953; Ozeki & Grundfest, 1965). At low temperature, the untreated fibres sometimes become capable of producing small spike potentials, an example of which is shown by inset *a* in Fig. 4*A* (obtained at 7° C in normal Tris-saline). Inset *b* shows that procaine at 0·05 % increased the spike overshoot of this fibre markedly.

The corresponding change in the current–voltage relation for the early transient current was a reduction in the amplitude of the outward current. The inward current was either unchanged or slightly increased. The membrane potential at which the early transient current reversed its sign shifted in the positive direction, corresponding to the increase in the overshoot of spike potential in the absence of voltage clamp. The slightly larger maximum value at the peak inward current found with procaine (Fig. 4*A*) corresponds to the finding that the maximum rate of rise of the spike occasionally shows a slight increase after procaine (Hagiwara & Nakajima, 1966*b*). The current–voltage relation at the peak of the early transient current was almost linear at the reversal potential. The slope of the curve at this potential should be roughly equal to the conductance of the membrane at the peak of the spike potential. The finding that procaine reduces this slope suggests that the spike potential under procaine is associated with an abnormally small increase of the membrane conductance even though its overshoot and maximum rate of rise are larger than their control values. A single explanation of these results is that procaine at low concentration suppresses the early K current but not the early Ca current. Since the reversal potential represents the potential at which the two currents cancel each other, the reduction in the K current should shift this potential in the positive direction. The reduced K current should increase the maximum inward current in the current–voltage relation even when the inward Ca current itself stays unchanged. The steady-state current was suppressed by procaine, corresponding to the prolongation of the spike potential sometimes found in the absence of voltage clamp.

It has been reported that some transition metal ions such as Co^{2+} or Mn^{2+} suppress the spike potential of barnacle muscle fibres (Hagiwara & Nakajima, 1966*b*; Hagiwara & Takahashi, 1967). Figure 4*B* shows the current–voltage relations of the membrane of the same fibre treated with Ca-chelating agent examined before and after addition of 30 mM-$CoCl_2$ in the normal Na-saline. A marked suppression of the early inward current but not of the early outward current was found. The result is very similar to that observed with conditioning depolarization. In other words, Co ions suppress the Ca current but not the early K current. The steady-state current usually showed no change when the Co^{2+} concentration was low (2–5 mM) even though the effect on the early inward current was already considerable. At a relatively high concentration, however, the steady-state current was also affected, as in the case of Fig. 4*B*. The major change was a shift of the current–voltage curve relation along the potential axis in the positive direction. This effect of divalent cations has been found in a number of different excitable tissues and is often called the stabilizing action on the membrane (Frankenhaeuser & Hodgkin, 1957).

Magnitude of the early K current

Figure 5 shows the current–voltage relations obtained in the normal Tris-saline with (stars) and without (filled circles) a conditioning depolarization. The conditioning resulted in a large reduction in the initial inward current. The fact that the two curves almost coincide at +100 mV indicates that the Ca current becomes negligible at approximately this mem-

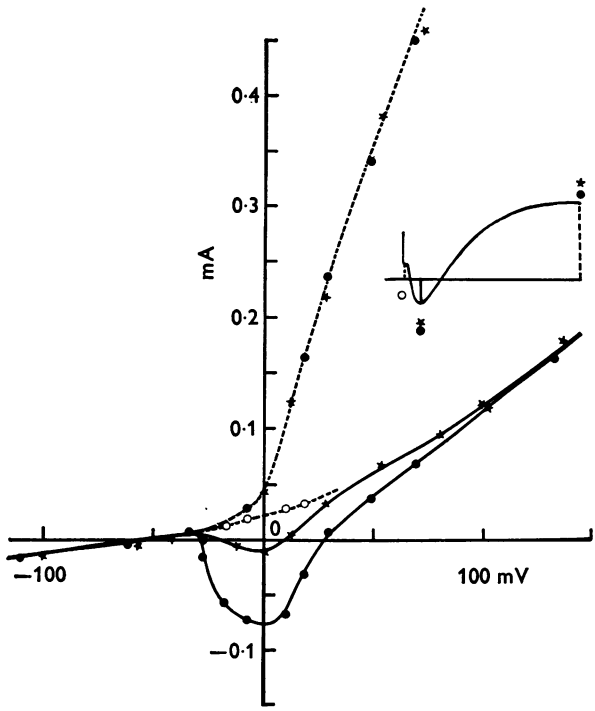

Fig. 5. Current–voltage relations at the peak of the early transient current (continuous lines) and at the steady state (interrupted lines, obtained at 308 msec after the onset of the test pulse). Values were obtained from the same muscle fibre at the resting membrane potential (−50 mV control) with (stars) and without (filled circles) conditioning depolarization (136 msec at −22 mV). Open circles represent the membrane current before excitation (see text and insert of this Figure). External solution, normal Tris-saline. Treated with the Ca-chelating internal solution. Temperature 8° C.

brane potential even without suppression. This suggests that the initial current obtained after strong suppression of the Ca current by conditioning represents essentially nothing but the K current when the membrane potential is not much lower than +100 mV.

Immediately after the onset of the voltage pulse the Ca current has not

yet been developed. Therefore, the membrane current at this time represents the current before excitation and should be carried mainly by K ions (probably partly by Cl ions also). This current is, however, difficult to determine because of the presence of capacitative current. As described before, the early inward current appeared at -35 to -40 mV. When the membrane potential was not much more positive than this value, the early inward current developed with a relatively slow time course (see traces for 26 mV and 48 mV in Fig. 2), and therefore the current at the end of the capacitative current (about 2 msec after the onset of the voltage pulse) does not seem to be much different from the current immediately after the onset of the pulse. Hence, the membrane current at the end of the capacitative current was taken as the current before excitation for this range of membrane potential (in the case of Fig. 5 up to $+20$ mV), and plotted against the membrane potential (Fig. 5, open circles). If the early conductance increase is not only to Ca ions but also to K ions, the K component of the early current should be greater than the membrane current found before the excitation. In the present case the magnitude of the early K current was not known for the range of membrane potentials at which the current–voltage relation before excitation was observed. However, the extrapolation of the current–voltage relation before excitation to a range of more positive membrane potentials suggests that the membrane current before excitation does not differ significantly from the early outward current obtained under strong suppression of the Ca current by conditioning depolarization. As discussed above, with large positive membrane potentials the total early current obtained under strong suppression is likely to represent almost exclusively the early K current. This leads to a conclusion that the early conductance increase occurs only to Ca ions but not to K ions.

Effect of Ca concentration

An increase in the external Ca concentration, by replacing a part of the NaCl (or Tris-Cl) in the normal solution with an osmotically equal amount of $CaCl_2$, results in an increase of the spike overshoot. This is always associated with a positive shift of the threshold membrane potential for spike generation. This shift is considered a result of the stabilizing action of Ca ions, seen under voltage clamp conditions as a shift of the current–voltage relation along the potential axis (Hagiwara, 1966). If the Ca concentration is varied in the presence of a relatively large amount of Mg ions, for example 100 mM-$MgCl_2$, the threshold membrane potential becomes independent of the Ca concentration at least in the range between 10 and 100 mM (Hagiwara & Takahashi, 1967). The three traces in the inserts of Fig. 6 were obtained at 10, 25 and 100 mM-Ca in the presence of

100 mM-Mg. The current–voltage relations obtained with voltage clamp show no shift along the potential axis upon the change in the Ca concentration. Steady current–voltage relations for three cases were identical. The amplitude of the early inward current decreased with decreasing Ca concentration while that of the early outward current at large positive membrane potentials was practically unchanged. This result can easily be explained in terms of the two component currents, i.e. the change in the external Ca concentration alters the Ca current but not the K current.

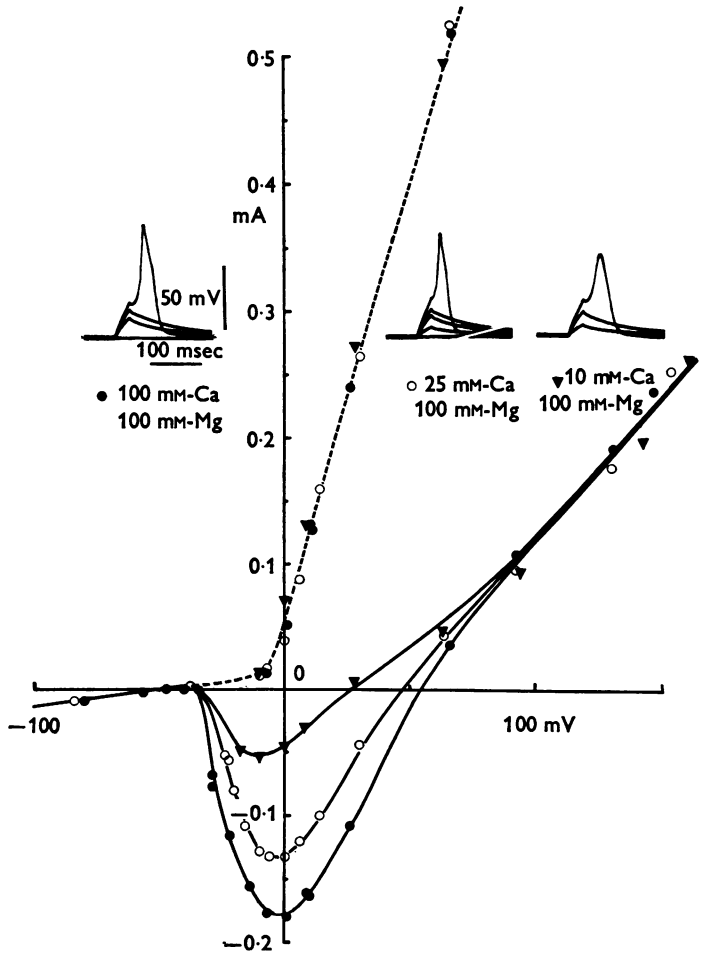

Fig. 6. Current–voltage relations at the peak of the early transient current (continuous lines) and at the steady state (interrupted lines, obtained at 145 msec after the onset of test pulse). Insert, spike potentials obtained in the absence of voltage clamp. External solution contained 100 mM-Mg and 100 mM-(filled circles), 25 mM-(open circles) and 10 mM-Ca (filled triangles). Treated with Ca-chelating internal solution. Temperature 9° C.

DISCUSSION

The experimental results show that membrane currents during a voltage clamp of a barnacle muscle fibre can be explained by a dual conductance model similar to that proposed for a squid giant axon (Hodgkin & Huxley, 1952*c*). In this case, however, the early conductance channel in the model passes Ca ions instead of Na ions. The late conductance channel in the barnacle fibre model appears to involve K ions just as it does in the squid axon model. The present work is not concerned with details of the late conductance channel, however.

The Na ion concentration inside the squid axon is significant even though it is smaller than that on the outside. Therefore Na ions can carry an inward as well as outward current of a significant amplitude through the early conductance channel. In a barnacle fibre, the internal Ca ion concentration is so small that no appreciable outward current can be carried by Ca ions. The internal Ca ion concentrations of the normal untreated fibre is usually in the range between 2×10^{-7} and 8×10^{-7} M and it is necessary to reduce the concentration below 8×10^{-8} M by injecting a chelating agent in order to obtain all-or-none spike potentials (Hagiwara & Nakajima, 1966*a*). Outward currents through the early conductance channel must be carried by other ions, namely by K ions. Chandler & Meves (1965) have concluded that the early conductance channel in the squid axon should pass not only Na ions but also other alkali metal ions such as K^+, Rb^+, etc. Their conclusion is based on the following two experimental facts obtained with axons perfused with Na-free solutions containing other alkali cations: (*a*) the early outward current during a voltage clamp is larger than that expected from the late conductance channel; and (*b*) the early outward and inward current are both suppressed equally well by a conditioning depolarization. In barnacle muscle fibres, the experimental results show that the early outward current during voltage clamp is not significantly larger than that expected from the resting membrane conductance and that the early outward current is insensitive to conditioning depolarization even when the same conditioning suppresses the early inward current. These findings lead to a conclusion that the early channel in the barnacle model does not pass alkali metal ions. Hence the reversal potential of the early transient current or the membrane potential at the peak of spike obtained without voltage clamping should be determined by the peak inward current through the early conductance channel and the outward current through the resting conductance channel or the late channel at the time when the early current reaches the peak. In other words, the peak membrane potential of the spike is not determined only by the early conductance channel.

As described before the early conductance channel in the squid axon model should pass various alkali cations (Chandler & Meves, 1965; Moore, Anderson, Blaustein, Takata, Lettvin, Pickard, Bernstein & Pooler, 1966). The experimental results of Watanabe & Tasaki (1967) suggest that it should also pass Ca^{2+}, Sr^{2+} and Ba^{2+}. The conductance increase is always suppressed by tetrodotoxin at a very low concentration (less than 10^{-7} g/ml., Rojas & Atwater, 1967; Watanabe & Tasaki, 1967). The early conductance channel in the barnacle muscle passes Ca, Sr and Ba ions (Hagiwara & Naka, 1964) but not alkali metal cations and the conductance increase is not suppressed by tetrodotoxin even when the concentration is as high as 10^{-4} g/ml. (Hagiwara & Nakajima, 1966*b*). This may suggest different molecular structures of the two sorts of membrane.

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